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Post-incision Steps of Nucleotide Excision Repair in *Escherichia coli*

DISASSEMBLY OF THE UvrBC-DNA COMPLEX BY HELICASE II AND DNA POLYMERASE I*

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UvrA, UvrB, and UvrC initiate nucleotide excision repair by incising a damaged DNA strand on each side of the damaged nucleotide. This incision reaction is substoichiometric with regard to UvrB and UvrC, suggesting that both proteins remain bound following incision and do not "turn over." The addition of only helicase II to such reaction mixtures turns over UvrC; UvrB turnover requires the addition of helicase II, DNA polymerase I, and deoxynucleoside triphosphates. Column chromatography and psoralen photocross-linking experiments show that following incision, the damaged oligomer remains associated with the undamaged strand, UvrB, and UvrC in a post-incision complex. Helicase II releases the damaged oligomer and UvrC from this complex, making repair synthesis possible; DNase I footprinting experiments show that UvrB remains bound to the resulting gapped DNA until displaced by DNA polymerase I. The specific binding of UvrB to a psoralen adduct in DNA inhibits psoralen-mediated DNA-DNA cross-linking, yet promotes the formation of UrvB-psoralen-DNA cross-links. The discovery of psoralen-UvrB photocross-linking offers the potential of active-site labeling.

Nucleotide excision repair eliminates DNA damage by removing an oligonucleotide from the strand containing the damage. The entire excision repair process can be divided into four steps: 1) incision/excision, which involves the scission of a phosphodiester bond on each side of the lesion; 2) removal of the oligomer containing the damaged nucleotide; 3) synthesis of a repair patch to fill the resulting gap in the DNA; and 4) ligation of the newly synthesized repair patch. In *Escherichia coli*, the incision step is catalyzed by the coordinated action of the *uvrA*, *uvrB*, and *uvrC* gene products (for reviews, see Sancar and Sancar (1988), Van Houten (1990), and Selby and Sancar (1990)). Specifically, this enzymatic action (known as (A)BC excinuclease) results in the incision of the damaged strand at both the eighth phosphodiester bond 5′ and the fourth or fifth phosphodiester bond 3′ to the lesion (Sancar and Rupp, 1983). The extent of incision, however, appears to be limited *in vitro* because one or more of the Uvr proteins do not dissociate after the incisions occur (Husain *et al.*, 1985). *In vivo* (Youngs and Smith, 1973; Van Sluis *et al.*, 1974; Kuemmerle and Masker, 1980; Tang and Smith, 1981) and *in vitro* (Kumura *et al.*, 1985; Caron *et al.*, 1985; Husain *et al.*, 1985) experiments have indicated that the *polA* (DNA polymerase I) and *uvrD* (helicase II) gene products are involved in post-incision events of nucleotide excision repair, including turnover of the UvrA, UvrB, and UvrC proteins; excision of the damage; and repair synthesis.

The current model for the steps leading to the incision of damaged DNA is as follows (Orren and Sancar, 1989, 1990). By way of a $(UvrA)_2$-$(UvrB)_1$ complex, UvrA delivers UvrB to a lesion in DNA; UvrA then must dissociate before UvrC can interact with the damage-specific UvrB-DNA complex to trigger the dual incisions in the damaged strand (Bertrand-Burggraf *et al.*, 1991). This model predicts that UvrA should turn over without the addition of DNA polymerase I (pol I)[1] or helicase II and that the lack of turnover of the excision nuclease *in vitro* is presumably due to the stable binding of UvrB and/or UvrC after incision occurs; release of these subunits by the combination of pol I plus helicase II leads to catalytic action of the (A)BC excinuclease.

In this study, we have examined the effects of helicase II and pol I on the post-incision structure of DNA and on the turnover of the UvrA, UvrB, and UvrC proteins. As predicted by the model, we found that UvrA can lead to >40 times more than a stoichiometric number of incisions without the addition of helicase II or pol I; under UvrA limiting conditions, neither the rate nor the extent of the incision reaction is influenced by helicase II and pol I. In contrast, both helicase II and pol I are necessary to efficiently turn over UvrB, which remains bound to DNA at the damaged site after incision and even after removal of the 12–13-mer containing the damage. Helicase II alone, however, can release both UvrC and the 12–13-mer from the damaged site.

MATERIALS AND METHODS

Enzymes, DNA Substrates, and Buffers—The Uvr proteins were purified as described (Thomas *et al.*, 1985). Helicase II was a kind gift of J. George and S. Matson (University of North Carolina). DNA polymerase I and T4 DNA ligase were purchased from Promega Biotec or Bethesda Research Laboratories, and pyruvate kinase was purchased from Sigma. Deoxyribonuclease I (DNase I) and T4 polynucleotide kinase were from Bethesda Research Laboratories. Antibodies to pol I were kindly provided by T. Ruscitti and S. Linn (University of California, Berkeley).

Unlabeled and ^{3}H-labeled plasmid DNAs were purified from *E. coli* strains AB2487/pBR322 and CH296/pDR3274 (Sancar and Rupp, 1983) by two consecutive CsCl/EtBr gradient centrifugations. When indicated, the plasmids were treated with UV light (254 nm) from a Sylvania germicidal lamp. A 138-bp DNA substrate containing a single, centrally located psoralen monoadduct was assembled from oligomers as described (Van Houten *et al.*, 1987), with the ^{32}P label

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[1] The abbreviations used are: pol I, DNA polymerase I; bp, base pair(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; dNTPs, deoxynucleotide triphosphates; SDS, sodium dodecyl sulfate.

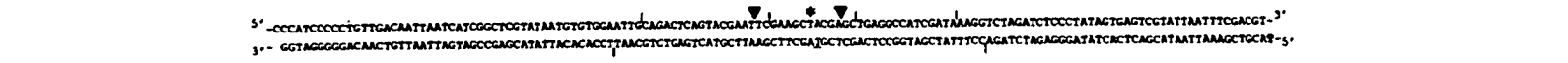

FIG. 1. **Psoralen-monoadducted substrate.** This DNA substrate was assembled from eight oligomers. The ligation sites between the oligomers are marked by *vertical bars*. The *asterisk* marks the position of the psoralen-adducted thymine; upon irradiation (365 nm), interstrand cross-links are formed between this adducted thymine and the adjacent thymine (*underlined*) on the undamaged strand. The 5′-^{32}P-labeled nucleotides on either the central, psoralen-adducted oligomer (internally labeled) or the 5′ end of the undamaged strand are in *boldface type*. The incision sites of (A)BC excinuclease on this substrate are indicated with *arrowheads*. Note that the dual incision of the internally labeled DNA by (A)BC excinuclease releases the label in the form of a 12-mer.

being incorporated either internally in the damaged strand or at the 5′ terminus of the undamaged strand. To examine release of the damage-containing oligomer, the psoralen-adducted central oligomer (12 nucleotides) was 5′ end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol) before annealing and ligating with other oligomers phosphorylated with unlabeled ATP. This DNA substrate, which contains the label at the sixth phosphate 5′ to the adduct, will be referred to as "internally labeled." For footprinting studies, the oligomer located at the 5′ end of the nonadducted strand was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and then annealed and ligated with the other oligomers as described above. The psoralen substrate is shown in detail in Fig. 1.

Reactions were done in ABC buffer (50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 5 mM dithiothreitol, and 20% glycerol), turnover buffer (ABC buffer supplemented with bovine serum albumin (50 μg/ml) and all four dNTPs (25 μM each)), and repair synthesis buffer (40 mM HEPES (pH 7.8), 50 mM KCl, 8 mM $MgCl_2$, 4% glycerol, 5 mM dithiothreitol, 100 μg/ml bovine serum albumin, 6% (w/v) polyethylene glycol 6000, 2 mM ATP, 40 μM each dATP, dGTP, and dTTP, and 4 μM dCTP plus 5 μCi of [α-^{32}P]dCTP (6000 Ci/mmol; Du Pont-New England Nuclear) per reaction). All buffers for column chromatography contained 10 mM β-mercaptoethanol instead of dithiothreitol.

Incision Assay—The incision assay measures nicking of superhelical plasmid DNA by analyzing the reaction products on agarose gels (Sancar *et al.*, 1984). UvrA, UvrB, UvrC, helicase II, pol I, and pol I antibodies were added as indicated to UV-irradiated, ^{3}H-labeled pBR322 DNA in turnover buffer and incubated at 37 °C for the indicated times. Reactions in which UvrA was the limiting protein also contained an ATP-regenerating system (phosphoenolpyruvate (250 μg/ml) and pyruvate kinase (1 unit/ml)). Alternatively, UvrB-DNA complexes purified by gel exclusion chromatography in low salt (100 mM KCl) buffer as described (Orren and Sancar, 1990) were supplemented with UvrC, helicase II, pol I antibodies, and/or pol I as indicated and incubated at 37 °C for the indicated times. All incision assay reactions were stopped by adding SDS to a final concentration of 0.2%. The DNA products were separated on 1% agarose gels; the bands corresponding to both superhelical and relaxed plasmids were excised; and the amount of each form was quantified by scintillation counting. The average number of incisions per plasmid was calculated from the fraction of superhelical molecules remaining assuming Poisson distribution of incisions.

Repair Synthesis Assay—UV-irradiated (225 J/m^2) pDR3274 was incubated at 1.3 nM (plasmid) in repair synthesis buffer (25 μl) with UvrB (100 nM), UvrC (70 nM), pol I (2 units), and T4 DNA ligase (1.2 units). When included, UvrA was at 4 nM, and helicase II was at 5 nM. After 25 min at 37 °C, the DNA was extracted with phenol and ether, precipitated in ethanol, and digested with *BglI*; and the 1230-bp fragment was resolved on a 3.6% sequencing gel. The gel was dried, and isotope incorporation was examined by autoradiography.

Separation of Reaction Intermediates by Column Chromatography—UvrA (5 nM), UvrB (170 nM), and UvrC (25 nM) plus or minus helicase II (4 nM) and/or pol I (0.25 unit) were incubated in turnover buffer (100 μl) for 15 min at 25 °C. When purified pol I was not added to the reaction, pol I antibodies (0.2 μg) were added to inhibit any contaminating pol I activity that might be present in the other proteins used. Internally labeled substrate was then added, and the reaction mixture was incubated for 30 min at 37 °C. A small aliquot (5 μl) was removed to determine the amount of incision, and the remainder of the reaction mixture was loaded onto an AcA202 (exclusion limit = 15 kDa) column (18 × 1 cm) equilibrated at 25 °C with ABC buffer. The column was developed with the same buffer; 500-μl fractions were collected; and radioactivity (DNA) in each fraction was measured by scintillation counting. The DNA in each fraction was precipitated with ethanol using oyster glycogen (100 μg) as carrier and then separated on 12% polyacrylamide sequencing gels that were subsequently dried and autoradiographed.

Alternatively, the reaction mixtures were loaded onto a blue Sepharose column (8 × 0.5 cm) equilibrated at 25 °C with the ABC buffer (containing 50 mM KCl) used above. The column was washed with the same buffer and then developed with ABC buffer containing 1.0 M KCl. Fractions of 300 μl were collected and analyzed for radioactivity and DNA content as described above.

Psoralen-mediated DNA-Protein and DNA-DNA Cross-linking—UvrA (5–50 nM) and/or UvrB (120 nM) was incubated with internally labeled substrate in turnover buffer (20 μl) for 30 min at 37 °C, and then unlabeled pBR322 DNA (0.6 nM) was added as indicated. UvrC (110 nM) and/or helicase II (4 nM) was added as indicated, and the reactions were incubated for an additional 30 min at 37 °C before irradiating with 365 nm light (2 milliwatts/cm^2) from a Spectroline Model B-100 black light lamp for 30 min at 25 °C. Some reaction mixtures were irradiated before the addition of UvrC.

The irradiated samples were analyzed for formation of DNA-protein or DNA-DNA interstrand cross-links by the following methods, respectively. 1) SDS loading dyes (25 μl) were added; and the sample was heated at 65 °C for 5 min and then loaded onto an SDS-6% polyacrylamide gel. The gel was silver-stained to visualize the Uvr protein markers and then dried and subjected to autoradiography. To obtain markers for proteins cross-linked to a 12-mer, UvrB (4 μM) or UvrC (9 μM) plus ~0.1 μM 5′-labeled, central 12-mer (single-stranded) containing psoralen were incubated and irradiated as described above. UvrC even at this high concentration did not photo-cross-link to the psoralen-adducted oligomer. 2) DNA products in the sample were ethanol-precipitated using oyster glycogen (100 μg) as carrier and then separated on sequencing gels (12%) that were subsequently dried and analyzed by autoradiography.

DNase I Footprinting—UvrA (5 nM) and/or UvrB (65 (nM) was incubated in turnover buffer (80 μl) at 37 °C for 30 min with the psoralen-adducted 138-mer labeled on the undamaged strand, and then unlabeled pBR322 DNA (0.7 nM) was added. The reaction mixtures were supplemented with UvrC (110 nM) and/or helicase II (4 nM) and incubated for an additional 10 min at 37 °C. Half of each sample was adjusted to 5 mM $CaCl_2$, supplemented with DNase I (100 pg), and incubated for 9 min at 25 °C; the other half was extracted with phenol, and the DNA was precipitated with ethanol and then treated with DNase I (400 pg) as described above. For both sets of samples, the DNase I digestion was stopped by the addition of EDTA (50 mM), and DNA was precipitated with ethanol using oyster glycogen (100 μg) as carrier. The DNA products were then separated on sequencing gels (8%) and visualized by autoradiography.

To measure (A)BC excinuclease incision of the psoralen-adducted DNA under these conditions, reactions containing UvrA, UvrB, UvrC, and unlabeled pBR322 DNA as described above were supplemented with internally labeled substrate before the initial 30-min incubation. The level of incision of internally labeled substrate was followed by analysis of the DNA products on sequencing gels.

RESULTS

Turnover of Uvr Proteins

It has been found that when equimolar concentrations of UvrA, UvrB, and UvrC are used in an incision reaction, no more than stoichiometric incisions can be achieved (Husain *et al.*, 1985). It was also reported that both pol I and helicase II are needed for enzymatic turnover of (A)BC excinuclease. However, it is now clear that the Uvr proteins do not act together as a multiprotein complex to incise DNA, but instead act in an ordered sequential manner (Orren and Sancar, 1989, 1990). In light of these new findings, we decided to re-examine the turnover of each Uvr protein individually.

Turnover of UvrA—According to the current model for the steps leading to the incision of damaged DNA (Orren and Sancar, 1989), $(UvrA)_2$ delivers UvrB to damaged sites in DNA and then must dissociate (Bertrand-Burggraf *et al.*, 1991) before UvrC can bind to the UvrB-damaged DNA complex and trigger the dual incisions in the damaged strand. This model thus predicts that a UvrA dimer should be able to catalytically deliver many molecules of UvrB to multiple sites on DNA, resulting in a greater number of incision events (upon addition of excess UvrC) than UvrA dimers present; it also predicts that addition of helicase II and/or pol I should not affect the turnover of UvrA. Fig. 2 (*upper*) shows the incision kinetics over an extended time period (4 h) in a reaction containing excess amounts of UvrB and UvrC, but limiting amounts of UvrA. After 4 h, ~40-fold more incisions have occurred than UvrA dimers present in the reaction mixture. These data conclusively demonstrate that UvrA is indeed acting catalytically in steps leading to the incision of damaged DNA. As expected, the addition of helicase II and pol I has little or no effect on either the rate or extent of incision under these conditions (Fig. 2, *lower*; also data not shown). From the early time points of Fig. 2 (*lower*), we estimate that the turnover rate for UvrA (as part of a $(UvrA)_2$/$(UvrB)_1$ complex) is ~1 mol of photoproduct bound per min/mol of $(UvrA)_2$-$(UvrB)_1$ complex (0.016 s^{-1}). We previously reported that the second order rate constant for formation of the $(UvrA)_2$-$(UvrB)_1$ DNA complex is ~6×10^4 M^{-1} s^{-1} (Orren and Sancar, 1990). Thus, it appears that the slow turnover of UvrA under these conditions is due to a slow rate of dissociation of $(UvrA)_2$ from the $(UvrA)_2$-$(UvrB)_1$-DNA complex.

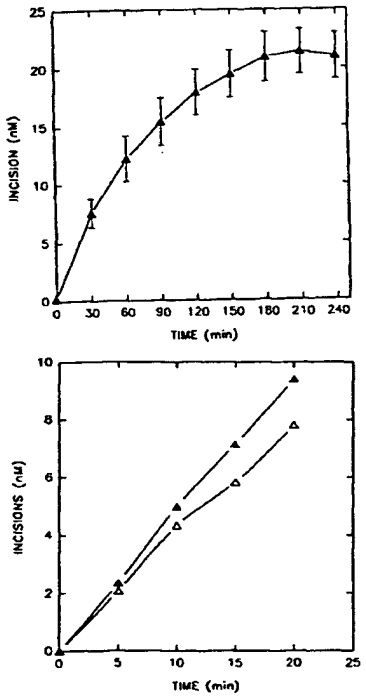

FIG. 2. **Incision of DNA using substoichiometric concentration of UvrA.** *Upper*, reaction mixtures containing UvrA (1 nM), UvrB (500 nM), UvrC (110 nM), and UV-irradiated (250 J/m²), ³H-labeled pBR322 DNA (9 nM) in turnover buffer (100 μl) containing the ATP-regenerating system were incubated at 37 °C. At 30-min intervals, aliquots (10 μl) were removed, the reaction was stopped, and the amount of incision was measured as described under "Materials and Methods." Each data point is the mean of three experiments, and the standard deviation is indicated by *error bars*. The background incision (linear for the duration of the experiment and reaching 4.9 nM at 240 min) resulting from the combination of UvrB, UvrC, and pyruvate kinase has been subtracted. *Lower*, reaction mixtures containing UvrA (1 nM), UvrB (500 nM), and UvrC (110 nM) without (△) or with (▲) pol I (0.25 unit) and helicase II (4 nM) in turnover buffer (100 μl) containing the ATP-regenerating system were incubated for 15 min at 25 °C. UV-irradiated (1000 J/m²), ³H-labeled pBR322 DNA (9 nM) was then added, and the reaction mixtures were incubated at 37 °C. At 0, 5, 10, 15, and 20 min, aliquots (10 μl) were removed and treated as described above. The data points are from one representative experiment. The background incision ranged from 0.5 nM at 5 min to 1.2 nM at 20 min for both reactions and has been subtracted from the data points.

If UvrB remains in the post-incision complex, we would expect that under UvrB limiting conditions, no more than a stoichiometric number (with regard to UvrB) of incisions can be made. We tested this prediction by incubating UV-irradiated pBR322 DNA and a limiting amount of UvrB with catalytic and excess amounts of UvrA and UvrC, respectively, and measuring incision. Helicase II, pol I, and dNTPs individually or in combinations were added to some of the reactions to evaluate their effects on incision. The results of these experiments are compiled in Table I. In the absence of pol I and helicase II, 0.16–0.3 nick/UvrB is produced. Since the stoichiometry of UvrB to DNA lesions is 1:1 (Orren and Sancar, 1989), less than stoichiometric incision probably reflects the fact that only a fraction of UvrB molecules present

Turnover of UvrB and UvrC—Filter binding (Husain *et al.*, 1985; Yeung *et al.*, 1986) and DNase I footprinting (Van Houten *et al.*, 1987) experiments indicate that a stable protein-DNA complex persists at the damaged site even after the dual incision of the damaged strand. Our model (and the data presented above) implies that this post-incision complex cannot contain UvrA, but must contain UvrB and/or UvrC. Earlier experiments on the turnover of the Uvr proteins suggest that both UvrB and UvrC remain bound following incision (Caron *et al.*, 1985; Husain *et al.*, 1985).

TABLE I

Effect of helicase II and pol I on the turnover of UvrB

Reaction mixtures containing UvrA (5 nM), UvrB (20 nM), UvrC (50 nM), helicase II (hel II) (4 nM), pol I (0.5 unit), and/or pol I antibodies (Ab) (0.2 μg) as indicated in turnover buffer (20 μl) were incubated for 15 min at 25 °C. UV-irradiated (250 J/m²), ³H-labeled pBR322 DNA (4.5 nM) was then added, and the mixtures were incubated for 30 min at 37 °C. After stopping the reactions, the superhelical and relaxed plasmid DNAs were separated by agarose gel electrophoresis, and the amount of incision was determined. For each experiment, the amount of incision for each sample was normalized to the amount of incision that was catalyzed by UvrA, UvrB, and UvrC alone (100%) in the same experiment (ranged from 0.7 to 1.3 nicks/molecule). The data are the mean ± S.D. of three experiments. We have observed minor nick translation activity in some of our Uvr proteins. To eliminate the contribution of possible contaminating pol I activity, we added pol I antibodies (which inhibited nick translation >90%) to the reaction mixtures when we wished to examine the effect of helicase II alone.

Additions and subtractions	Activity
	%
UvrA, UvrB, and UvrC	100.0
+Ab	96.0 ± 6.4
+pol I	90.0 ± 4.1
+pol I + Ab	100.0 ± 9.6
+hel II	133.7 ± 12.5
+hel II − dNTPs	109.3 ± 4.8
+hel II + Ab	123.3 ± 1.7
+hel II + Ab − dNTPs	107.7 ± 3.7
+hel II + pol I	174.7 ± 17.2
+hel II + pol I − dNTPs	128.3 ± 5.4
+hel II + pol I + Ab	121.3 ± 5.3
+hel II + pol I + Ab − dNTPs	112.3 ± 6.6

in our preparations are active or that only a fraction of the UvrB-DNA complexes are productive complexes that lead to incision upon adding UvrC. Alone, pol I (in the presence or absence of dNTPs) does not stimulate the level of incision. However, when both helicase II and pol I are added in the presence of dNTPs, the level of incision is optimally enhanced. Withholding dNTPs reduces the level of incision to that comparable to the reaction with helicase alone added, suggesting that repair synthesis (and not just binding) by pol I is necessary for complete release of UvrB from incised sites. The small amount of stimulation by helicase II alone could be due to UvrC turnover (see below), to decreased binding stability of UvrB to the post-incision site after helicase II action, or to residual contaminating pol I activity even in the presence of pol I antibodies.

At present, there is no convincing physical evidence that UvrC remains bound at the damaged site after incision has occurred. Nevertheless, the stoichiometric relationship of incision to the amount of UvrC (Yeung *et al.*, 1986) and the lack of turnover in the absence of accessory proteins (Caron *et al.*, 1985; Husain *et al.*, 1985) are circumstantial evidence that the post-incision protein-DNA complex contains UvrC. To demonstrate that UvrC does not turnover after incision, we added limiting amounts of UvrC to UvrB-damaged DNA complexes purified by gel exclusion chromatography. We also tested the ability of helicase II and/or pol I to stimulate the level of incision catalyzed in the presence of limiting amounts of UvrC (Fig. 3). In the absence of helicase II and pol I, 0.25 incision/UvrC molecule was obtained, suggesting that 25% molecules are active in our UvrC preparation (assuming $(UvrB)_1:(UvrC)_1$ stoichiometry in the incision complex) (see Lin and Sancar (1991)). Helicase II (with or without pol I antibodies) stimulates this level of incision; in contrast, pol I alone has no effect on the rate or level of incision, and it does not further stimulate the helicase II-stimulated reaction (data not shown). Helicase II affects the extent but not the initial rate of UvrC-dependent incision, indicating that helicase II does not increase the efficiency of incision, but stimulates the turnover of UvrC (Fig. 3). In agreement with this observation, the patterns of stimulation of incision by adding helicase II or additional UvrC after incision has reached a plateau (in the presence of limiting UvrC) are similar (Caron *et al.*, 1985; data not shown), suggesting that helicase II releases UvrC from post-incision complexes. The release of UvrC may uncover the 5′ incision site (Lin and Sancar, 1991), enabling pol I to carry out repair synthesis.

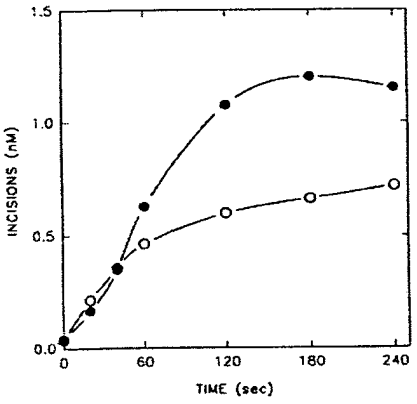

FIG. 3. **Effect of helicase II on rate and extent of incision in presence of limiting concentration of UvrC.** A reaction mixture containing UvrA (20 nM), UvrB (1.5 μM), and UV-irradiated (250 J/m²), ^{3}H-labeled pBR322 DNA (11 nM) in ABC buffer was incubated for 20 min at 37 °C and then diluted 2-fold with ABC buffer containing 0.1 M KCl. UvrB-DNA complexes were isolated by gel exclusion chromatography as described (Orren and Sancar, 1989), except the elution buffer contained 0.1 M KCl. The concentrations of UvrB and DNA in the isolated complexes were 6.6 and 0.7 nM (~7 nM photoproducts), respectively. UvrC (2 nM) without (○) and with (●) helicase II (4 nM) was added to aliquots (200 μl) of the isolated UvrB-DNA complexes; the reactions were incubated at 37 °C; and aliquots (25 μl) were removed at the indicated times. The incision reactions were immediately stopped, and the amounts of incisions were quantified. No background incision was detectable under the conditions used in this assay.

Dependence of Repair Synthesis on Helicase II

To measure the effect of helicase II on repair synthesis catalyzed by pol I, we incubated a UV-irradiated plasmid with a partial repair system (UvrB, UvrC, pol I, and dNTPs (including [α-^{32}P]dCTP)) plus or minus UvrA and/or helicase II. Following incubation of these mixtures, the plasmid was digested with a restriction enzyme, and the DNA products were separated on a polyacrylamide sequencing gel to estimate repair synthesis by autoradiography. The result is shown in Fig. 4. When UvrA was withheld from the reaction mixture, incision at damaged sites did not occur; and thus, there was no repair synthesis. We attribute the low level of DNA synthesis to nick translation by pol I. Interestingly, when the reaction mixture contained UvrA, but not helicase II, the level of DNA synthesis was approximately equal to that in the reactions that did not contain UvrA, indicating that repair synthesis still did not occur possibly because UvrC blocks access of pol I to the 5′ incision site. When both UvrA and helicase II were included in the reaction mixture, the level of DNA synthesis increased dramatically, indicating that helicase II, by releasing UvrC, permits repair synthesis by pol I at damaged sites. An earlier study reported stimulation by (but not strict dependence on) helicase II of repair synthesis in UV-irradiated plasmid (Kumura *et al.*, 1985). The difference between the earlier study and this one possibly stems from the relative purities of the Uvr proteins used in the two studies.

Release of Damaged Oligomer

The data presented above as well as a retrospective interpretation of earlier work (Caron *et al.*, 1985; Husain *et al.*, 1985) suggest that following the dual incisions, UvrB, UvrC, and the 12-mer containing the damaged nucleotide remain associated with the DNA substrate. However, no physical evidence exists for such a structure, and it is possible to propose alternative models consistent with the kinetic data. To obtain direct evidence, we probed the structure and composition of the post-incision complex by column chromatog-

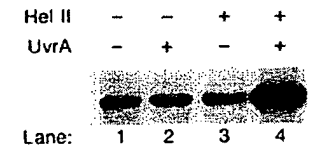

FIG. 4. **Repair synthesis requires DNA helicase II.** Repair synthesis reactions (25 μl) contained UV-irradiated (225 J/m²) pDR3274 DNA (1.3 nM), UvrB (100 nM), UvrC (70 nM), pol I (2 units), T4 DNA ligase (1.2 units), and, where indicated, UvrA at 4 nM and helicase II (*Hel II*) at 5 nM. Radiolabeled dCTP was included as the tracer. Following repair synthesis, the plasmid was digested with *BglI*; an autoradiograph of the 1230-bp fragment, resolved on a 3.6% denaturing polyacrylamide sequencing gel, is shown. Results with other restriction fragments were the same, but are omitted for clarity.

raphy and photocross-linking using internally labeled substrate, a 138-bp DNA fragment in which a radioactive label was incorporated into the sixth phosphodiester bond 5′ to a specifically located psoralen adduct (see Fig. 1).

To examine the excision and release of the psoralen-containing 12-mer from DNA, we used gel exclusion chromatography. The molecular masses of the 138-mer substrate and the 12-mer that results from dual incision of this substrate are approximately 90 and 4 kDa, respectively. A gel filtration column with an exclusion limit of 15 kDa allows separation of the free 12-mer from the 138-bp substrate or the gapped product that elutes in the void volume.

Reactions containing internally labeled substrate, UvrA, UvrB, UvrC, and pol I or pol I antibodies plus or minus helicase II were chromatographed; and fractions were analyzed for DNA content by polyacrylamide gel electrophoresis. The elution profiles are shown in Fig. 5. In the reaction containing only UvrA, UvrB, and UvrC (Fig. 5*A*), a fraction (30–40%) of the 12-mer eluted in the void volume with the unincised 138-mer, whereas the rest was slightly retarded, indicating that under these conditions, the damaged oligomer remains bound either to the incised DNA or to one of the Uvr proteins that also elutes in the void volume. The fact that some of the excised oligomer eluted in the included volume suggests that the oligomer is not that tightly bound in the post-incision complex and partly dissociates under this non-equilibrium condition. When UvrA, UvrB, UvrC, and helicase II were present in the reaction mixture (Fig. 5*B*), all of the excised oligomer eluted as the free 12-mer. When added to reactions with or without helicase II, pol I did not affect the elution profile or the distribution of free and bound 12-mers (data not shown). Thus, helicase II alone appears to be sufficient to completely release the damaged oligomer from the totally incised 138-bp substrate, UvrB, and UvrC.

The results presented above do allow us to distinguish whether the 12-mer that coelutes with the unincised substrate (in the absence or presence of pol I) is associated with the gapped 138-mer (nonradioactive) or is bound to either free UvrB or UvrC. Both UvrB and UvrC bind to blue Sepharose (Thomas *et al.*, 1985), but DNA did not (data not shown); so we would expect post-incision protein-DNA complexes to be retarded, whereas free DNAs pass directly through this type of column. Using this column, we found that most of the excised oligomer along with UvrB and UvrC remained bound to the column after low salt (50 mM KCl) wash and was eluted with high salt (1.0 M KCl) (data not shown). Helicase II in the reaction mixture caused the elution of the oligomer (but not of UvrB or UvrC) by low salt. pol I, alone or in combination with helicase II, had no effect on the elution patterns. Thus, the gel exclusion and affinity chromatography data together lead us to conclude the helicase II alone is sufficient to release the 12-mer in protein-free form.

DNA Interstrand Cross-linking in Pre- and Post-incision Complexes

More detailed information on the structure and composition of the post-incision protein-DNA complex and the effect of helicase II on this complex was obtained by psoralen cross-linking experiments. Psoralen has two photoreactive groups (Cimino *et al.*, 1985). Thus, the psoralen-thymine furan side monoadduct at the TA sequence in our substrate can be quantitatively converted to an interstrand cross-link with 365 nm irradiation (Gamper *et al.*, 1984) as shown in Fig. 6 (*lane 2*). The incubation of internally labeled substrate with UvrA or UvrB in the presence or absence of unlabeled pBR322 DNA before irradiation did not change the extent of interstrand cross-linking (*lanes 3–5*). In contrast, when both UvrA and UvrB were incubated with this substrate, the extent of cross-linking was severely inhibited (*lane 6*). Apparently, the conformational change (kinking, unwinding) of the DNA caused by binding of UvrB creates unfavorable geometry for

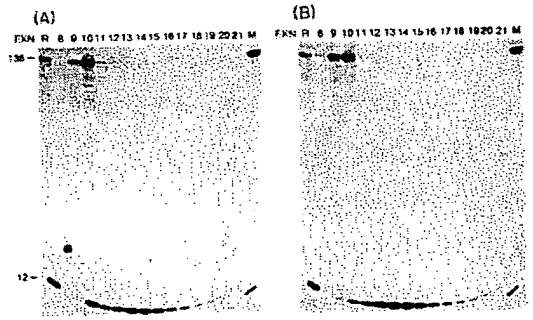

FIG. 5. **Release of excised oligomer by helicase II.** Internally labeled substrate was treated with (A)BC excinuclease without (*A*) or with (*B*) helicase II. A small fraction of each reaction mixture was electrophoresed in *lane R* of each gel. The remainder of each reaction mixture was chromatographed by gel filtration of AcA202, and the DNA products of fractions containing radioactivity were analyzed on 12% sequencing gels. *Lane M* of each gel contains untreated 138-bp substrate and the undamaged 12-mer as markers. The fraction numbers are indicated on each radioautograph as well as the positions of the unincised 138-mer and the excised 12-mer. Note that the psoralen-adducted 12-mer migrates slightly slower than the undamaged 12-mer. In the absence of proteins, the free 12-mer elutes in an identical manner to the elution profile of the 12-mer shown in *B*. *FXN*, fractions.

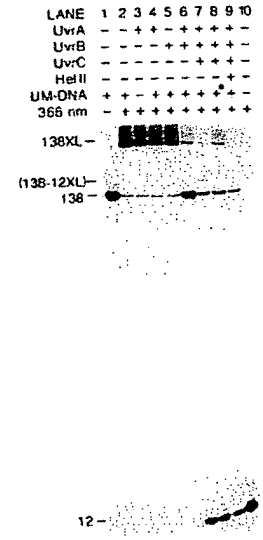

FIG. 6. **Effect of Uvr proteins on formation of psoralen interstrand cross-links.** Reactions that contained internally labeled psoralen substrate plus UvrA, UvrB, UvrC, helicase II (*Hel II*), and unlabeled pBR322 DNA (*UM-DNA*) as indicated were irradiated with 366 nm light where indicated to form DNA interstrand cross-links. The *asterisk* (*lane 8*) denotes that the reaction mixture was irradiated before the addition of UvrC. The DNA products from these reactions were separated on a 12% sequencing gel. The positions of the unincised cross-linked (*136XL*), incised cross-linked (*138-12XL*), unincised uncross-linked (*138*), and incised uncross-linked (*12*) DNA products are indicated. Unlabeled pBR322 DNA, by competing for free UvrA, favors the formation of the UvrB-DNA complex and improves incision efficiency (Bertrand-Burggraf *et al.*, 1991). *Lane 10* contains terminally labeled 12-mer standard.

the second photoaddition reaction. The addition of UvrA, UvrB, and UvrC prior to irradiation resulted in the generation of a labeled 12-mer as well as a unique band we attribute to the 12-mer cross-linked to the unlabeled undamaged strand (*lane 7*). This suggests that after incision, some or all of the damaged oligomer remains associated with the undamaged strand and thus is cross-linkable. This unique band almost completely disappeared if, prior to irradiation, helicase II was added to reactions containing UvrA, UvrB, and UvrC (*lane 9*). That not all of the excised oligomer was cross-linked to the complementary strand could mean either that only a fraction of the oligomer remains in the post-incision complex or that all remains bound, but because of the less favorable conformation of the post-incision complex compared to free DNA, the cross-linking is less efficient. When helicase II was added, most or all of the 12-mer was released from the undamaged strand and therefore could not be cross-linked.

Cross-linking of UvrB to Psoralen-adducted Substrate and Product

The unique photochemistry of psoralen also enabled us to probe directly the proteins bound in the pre- and post-incision complexes and their proximities to DNA. We reasoned that if a protein is closely associated with a DNA-psoralen monoadduct such as to inhibit its conversion to a DNA interstrand cross-link, the protein itself might be cross-linked upon irradiation at 365 nm. Therefore, we conducted experiments similar to those presented in Fig. 6, but analyzed the reaction products by SDS-polyacrylamide gel electrophoresis to detect DNA-protein cross-links.

Fig. 7 shows that when UvrA and UvrB were incubated with internally labeled substrate before irradiation, a unique band appeared that was not present in the control reactions containing either UvrA or UvrB (compare *lane 5* to *lanes 3–4*). This band almost completely disappeared when UvrC was added before irradiation; but another faster migrating, unique band appeared (*lane 6*). This band migrated slightly slower than UvrB (data not shown) and co-migrated with a band

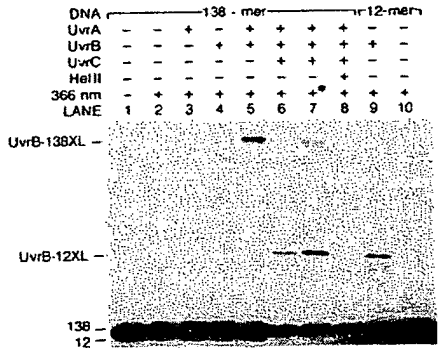

FIG. 7. **Cross-linking of UvrB to psoralen-adducted substrates.** Incision and photocross-linking reactions of mixtures containing the indicated proteins were carried out, and then the radioactive products were visualized by autoradiography after separation by SDS-6% polyacrylamide gel electrophoresis. The DNA substrate in the reactions was either the internally labeled 138-mer (*lanes 1–8*) or the 5′ end-labeled, psoralen-adducted 12-mer (*lanes 9* and *10*). In *lanes 3–8*, the concentrations of UvrA, UvrB, UvrC, and helicase II (*Hel II*) were 5, 120, 110, and 4 nM, respectively; *lane 9* contained the 12-mer (single strand) at ~0.1 μM and UvrB at 4 μM. The *asterisk* (*lane 7*) indicates that in this reaction, irradiation was done before the addition of UvrC. The positions of UvrB cross-linked to the 138-mer (*UvrB-138XL*) and to the 12-mer (*UvrB-12XL*) as well as the positions of the protein-free 138-mer (*138*) and the free 12-mer (*12*) are indicated.

from an irradiated control reaction in which only UvrB and the 5′ end-labeled, psoralen-adducted 12-mer were present at very high concentrations (*lane 9*). We conclude that the faster migrating unique band is UvrB cross-linked to the psoralen-adducted 12-mer generated by dual incision of the substrate. When UvrA, UvrB, UvrC, and helicase II were present in the reaction before irradiation, the faster migrating band completely disappeared (*lane 8*), indicating that helicase II physically separates the damaged 12-mer from UvrB so that cross-linking can no longer be achieved.

If the faster migrating band is UvrB cross-linked to the damaged oligomer, then the slower migrating band is UvrB cross-linked to the unincised 138-mer. This conclusion is supported by the experiment carried out in Fig. 7 (*lane 7*). The sample was irradiated after incubation of substrate with UvrA and UvrB, but before addition of UvrC. The presence of the UvrB-12-mer cross-link in this lane arose from a pre-incision complex that was cross-linked and then became incised upon addition of UvrC. Since we know that the cross-linked post-incision complex contains UvrB, the pre-incision complex that was cross-linked must have been UvrB bound to the 138-bp substrate. The fact that UvrB was cross-linked with about the same efficiency to both the 138-mer and the excision product indicates that all or most of the excised oligomer remains associated with UvrB. Interestingly, this control also demonstrates that photocross-linking of UvrB to the 138-mer via psoralen does not change the structure of either the protein or the damaged region enough to prevent incision upon addition of UvrC. Although unlikely, another possibility is that the UvrB-DNA cross-link is recognized and incised by uncross-linked active UvrA, UvrB, and UvrC proteins.

The column chromatography and psoralen cross-linking experiments provide physical evidence for the structure of the post-incision complexes. The fact that the 12-mer can be cross-linked to the undamaged strand confirms that the damaged oligomer remains as part of the post-incision complex. Cross-linking of UvrB to the excised oligomer indicates that before helicase II is added, the psoralen adduct is in intimate contact with UvrB. When helicase II is present, the damaged 12-mer is separated from both UvrB and the undamaged strand and cannot be cross-linked to either. In agreement with this conclusion, our chromatographic experiments show that the addition of helicase II to (A)BC excinuclease-treated substrates results in the release of the adducted 12-mer free of UvrB and UvrC. The turnover experiments indicate that UvrC is also released by helicase II, whereas UvrB remains bound to the "gapped" DNA resulting from excision. More definitive data for the existence of a UvrB-gapped DNA complex was obtained by DNase I footprinting.

DNase I Footprinting of Pre- and Post-incision Complexes

The existence of a post-incision DNA complex was observed by DNase I footprinting of the 138-bp psoralen substrate, labeled at the 5′ end of the undamaged strand (Van Houten *et al.*, 1987). In this study, this substrate was used to examine the pre- and post-incision complexes generated by the Uvr proteins plus or minus helicase II; specifically, the DNase I footprints of pre- and post-incision protein-DNA complexes were compared with those of the DNA after protein and the excised 12-mer were removed by phenol extraction. Our UvrB footprint (Fig. 8, *lane 4*) is identical to that observed previously (Van Houten *et al.*, 1987; Bertrand-Burggraf *et al.*, 1991). When UvrA, UvrB, and UvrC were added, ~75% of the substrate was incised (data not shown), and the footprint on the undamaged labeled strand spread slightly in the 3′

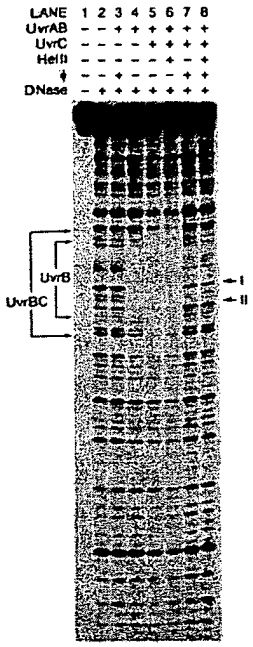

FIG. 8. **DNase I footprints of pre- and post-incision UvrB-DNA complexes.** Reaction mixtures containing psoralen-adducted substrate labeled in the undamaged strand and unirradiated unlabeled pBR322 DNA (0.7 nM) plus UvrA (5 nM), UvrB (65 nM), UvrC (110 nM), and/or helicase II (*Hel II*) (4 nM) were incubated with DNase I (except *lane 1*). Where indicated, phenol extraction (Φ) was carried out prior to treatment with DNase I. The regions of the undamaged strand protected by UvrB and the UvrB-UvrC complex are *bracketed*. *Bands I* and *II* are noted for comparison of the digestion patterns of unincised DNA (*lanes 2* and *3*) with those of incised gapped DNA (*lanes 7* and *8*).

direction and more significantly in the 5′ direction (*lane 5*) compared to the pre-incision UvrB footprint. When helicase II was added (*lane 6*), the bands at the 5′ and 3′ boundaries of the footprint reappeared to a slight extent, but not to the pre-incision level. Comparison of these footprints of post-incision complexes to the DNase I digestion patterns of samples treated with phenol after incubation with UvrA, UvrB, and UvrC plus or minus helicase II (*lanes 7* and *8*) confirms the existence of a protein-gapped DNA complex even after the addition of helicase II. Note that the DNase I digestion pattern for the gapped DNA is different from that of the unincised protein-free substrate (compare *bands I* and *II* in *lanes 2* and *3* to those in *lanes 7* and *8*). The similarity of the pre-incision UvrB footprint (*lane 4*) to the gapped DNA post-incision footprint (*lane 6*) suggests that the protein that remains bound is UvrB. The fact that helicase II alone will cause UvrC (but not UvrB) to turnover supports this conclusion. The difference in the pre- and post-incision footprints may arise from changes in the interaction of UvrB after incision occurs or from the effect of binding of UvrC (*lane 5*) or helicase II (*lane 6*). However, we cannot distinguish between these possibilities at this time.

A point of concern in interpreting experiments involving helicase II and linear DNA is that helicase II can unwind duplex DNA from a blunt end (Runyon and Lohman, 1989; Runyon *et al.* 1990). However, this only occurs at high protein:DNA rations and is undetectable under the conditions used here as apparent from the fact that helicase II-containing DNA samples (Fig. 8, *lanes 6* and *8*) are as sensitive to DNase I (and therefore in double-stranded form) as the corresponding samples without helicase II (*lanes 5* and *7*).

DISCUSSION

Previous studies have demonstrated the existence of protein-DNA complexes following incision by (A)BC excinuclease (Husain *et al.*, 1985; Yeung *et al.*, 1986) and the lack of turnover of the Uvr proteins *in vitro* (Caron *et al.*, 1985; Husain *et al.*, 1985). This study: 1) identifies the proteins present in post-incision complexes, 2) defines the minimal conditions necessary for turnover of each Uvr protein and release of the excised oligomer, and 3) links post-incision changes in DNA structure (*i.e.* release of incised oligomer, synthesis of a repair patch) to the turnover of the Uvr proteins.

According to the current model for the pre-incision steps of excision repair (Orren and Sancar, 1989), UvrA, which has specific affinity for damaged DNA, delivers UvrB (in the form of a $(UvrA)_2$-$(UvrB)_1$ complex) to damaged sites and dissociates from the complex. UvrC interacts with the UvrB-DNA complex and triggers incision of the damaged strand on both sides of the lesion. The dissociation of UvrA appears to be essential for the incisions to occur as incision is inhibited if UvrA remains bound at the damaged site (Bertrand-Burggraf *et al.*, 1991). Thus, the function of UvrA is to recognize damage in DNA and somehow make it possible for UvrB to bind to the damage (UvrB by itself binds DNA nonspecifically with very low affinity); since UvrA must dissociate before incision can occur, it should be able to deliver UvrB to damaged sites in a catalytic manner. Indeed, this was observed. Helicase II and pol I had no effect on the rate or extent of incision under UvrA limiting reaction conditions, a result which is in apparent contradiction to earlier conclusions based on experiments conducted with equimolar amounts of UvrA and UvrB at a time when it was thought that the incision complex contained all three subunits (Husain *et al.*, 1985; Caron *et al.*, 1985); thus, UvrB was probably the limiting factor in those experiments. As we will discuss below, both helicase II and pol I are necessary for optimal turnover of UvrB.

Our studies have convincingly demonstrated that a UvrA dimer can catalytically deliver many molecules of UvrB to damaged sites in DNA, resulting in a much greater number of incision events than UvrA dimers present. The *in vivo* ratios of UvrB to $(UvrA)_2$ (20:1 in uninduced and 8:1 in SOS-induced cells) (see Selby and Sancar (1990)) are consistent with this mechanistic role for UvrA. Because of its ability to bring two compatible yet reluctant components (UvrB and DNA) together, UvrA can be called a "molecular matchmaker." Transcription factors IIIA and IIIC, by virtue of their ability to deliver TFIIIB to its specific binding site upstream of the 5 S rRNA gene (Kassavetis *et al.*, 1990), and DnaK and DnaJ, which alter RepA protein to increase its affinity to the P1 origin of replication (Wickner *et al.*, 1991), might be considered other examples of molecular matchmakers. This contrasts with molecular chaperons that mediate the folding of certain proteins by preventing improper interactions between potentially complementary surfaces (Ellis and Hemmingsen, 1989). However, the distinction between the two classes of helper proteins may not be absolute as exemplified by the fact that DnaK functions in both capacities.

In the absence of helicase II and pol I, the post-incision protein-DNA complex must contain UvrB and/or UvrC. The less than stoichiometric incision observed when either UvrB or UvrC was present in limiting amounts suggests that both proteins remain bound to the incised DNA. In reactions where UvrC was the limiting protein, we found that only helicase II

was necessary to stimulate incision, in agreement with an earlier report (Caron *et al.*, 1985). Our chromatography experiments demonstrate that the addition of only helicase II to (A)BC excinuclease-treated substrates results in the complete release of protein-free, psoralen-adducted oligomer from the 138-bp substrate. This is consistent with the previously documented activity of helicase II on nicked DNA substrates (Runyon and Lohman, 1989; Runyon *et al.*, 1990). The psoralen cross-linking experiments confirm that helicase II releases the adducted oligomer. A cross-linked DNA product (excised oligomer linked to the undamaged strand) observed in (A)BC excinuclease-treated reactions disappears when those reactions are supplemented with helicase II. The existence of this cross-linked product also confirms that the damaged oligomer remains annealed following incision.

Similarly, cross-linking of UvrB to the psoralen-adducted oligomer is also eliminated by the addition of helicase II, indicating that the excised oligomer is no longer associated with UvrB. Thus, the role of helicase II is to release both UvrC and the damaged oligomer from the post-incision protein-DNA complex. This result contradicts an earlier report that suggested that both pol I and helicase II are necessary for release of the damaged oligomer following (A)BC excinuclease-mediated incision (Caron *et al.*, 1985). The helicase II-dependent removal of UvrC and the damaged oligomer to create a gapped DNA apparently is necessary for synthesis of the repair patch by Pol I as evidenced by the lack of repair synthesis in the absence of helicase II.

The similarities of the pre- and post-incision DNase I footprints observed earlier (Van Houten *et al.*, 1987) and in this study suggest that UvrB remains bound after incision occurs. In addition, the footprints of (A)BC excinuclease-treated substrate in the presence and absence of helicase II are nearly identical. Since we have determined that helicase II releases UvrC, the protein-DNA complex following helicase action must therefore contain UvrB. In accordance with this notion, both helicase II and pol I (in the presence of dNTPs) were necessary to optimally stimulate incision in reactions where UvrB was the limiting protein. The requirement for dNTPs implies that binding of pol I at the 5′ incision site is not sufficient to cause turnover of UvrB and that repair synthesis by pol I is necessary to displace UvrB from the gapped DNA.

Based on these results and the previous work on the steps leading to incision of damaged sites in DNA (Orren and Sancar, 1989, 1990; Bertrand-Burggraf *et al.*, 1991), we propose the following model for nucleotide excision repair in *E. coli* (Fig. 9). $(UvrA)_2$ associates with UvrB to form the $(UvrA)_2$-$(UvrB)_1$ complex in an ATP-dependent reaction; this complex scans DNA (Seeley and Grossman, 1990) and binds to the damage site; $(UvrA)_2$ is released, leaving behind a stable UvrB-DNA complex. UvrC associates with this complex, and the two incisions are made. A quaternary complex consisting of UvrB, UvrC, the excised oligomer, and DNA persists. Helicase II releases UvrC and the excised oligomer, resulting in a stable UvrB-gapped DNA complex. pol I binds to the 3′-OH end of the gap, which apparently is accessible to the enzyme (Van Houten *et al.*, 1988), fills in the gap, and dislodges UvrB. Under physiological conditions, pol I and helicase II might act in a concerted manner, dissociating the post-incision complex and filling in the gap simultaneously (Matson, 1986). Following repair synthesis, the nick is immediately sealed with ligase, preventing any significant nick translation; as a result, nearly all repair patches are 12–13 nucleotides in length (Sibghat-Ullah *et al.*, 1990).

UvrB at physiological concentrations does not bind signif-

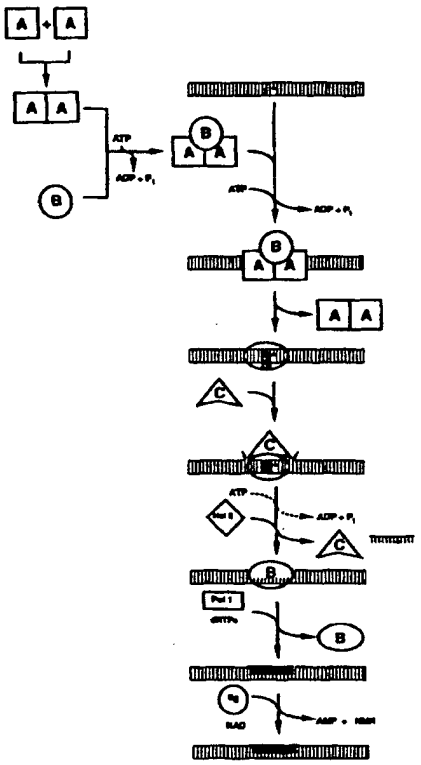

FIG. 9. **Model for nucleotide excision repair in *E. coli*.** The association of UvrA and UvrB and the loading of UvrB onto DNA require ATP hydrolysis. Incision by the UvrB-UvrC complex requires ATP binding; the incision sites are shown by *small arrows*. Displacement of UvrC and excised oligomer by helicase II (*Hel II*) probably requires ATP hydrolysis (indicated with *broken arrows*). Whether helicase II is directly involved in releasing UvrB is not known.

icantly to DNA in the absence of UvrA; however, the UvrB-damaged DNA complex formed after delivery by UvrA is extremely stable even in solutions of high ionic strength (Orren and Sancar, 1989). Thus, UvrA causes a change in either UvrB or the DNA (or both) that results in a highly specific, stable interaction. The molecular mechanism of the interaction between UvrB and the damaged site remains unclear, but must involve hydrophobic or intercalating types of interactions because of its extreme stability to high ionic strength. Indeed, an unexpected observation (UvrB-DNA cross-links) in our cross-linking experiments revealed that UvrB must be in intimate contact with the DNA both before and after the incisions occur.

In B-DNA, psoralen monoadducts in 5′-TA sequences are converted efficiently to DNA interstrand cross-links upon irradiation with 365 nm light (Cimino *et al.*, 1985; see also Fig. 5). However, when UvrB is bound to the psoralen-monoadducted substrate, interstrand cross-linking is drastically inhibited, indicating that the structure of the DNA in the vicinity of the psoralen monoadduct has been altered so that the reactive pyrone group of psoralen can no longer form a chemical bond to an adjacent thymine in the opposite strand. A possible explanation is that in the UvrB-damaged DNA complex, the DNA is severely kinked or the strands of DNA have been separated (Oh and Grossman, 1986); and simply, the larger distance between the strands at the adducted site prevents interstrand cross-linking. Perhaps the damage recognition reaction itself requires unwinding or kinking of the

DNA in the vicinity of the adduct, followed by the formation of a stable UvrB-unwound kinked DNA complex.

Finally, our observation of inhibition of interstrand cross-link formation by UvrB and the formation of UvrB-DNA cross-links should have more general applications in the field of DNA-protein interactions. Thus, the inhibition of interstrand cross-link formation by a bound protein might be a sensitive probe for structural changes in DNA brought about by binding. For example, even though $(UvrA)_2$ provides a complete protection from DNase I to a 33-bp region around the adduct (Van Houten *et al.*, 1987; Bertrand-Burggraf *et al.*, 1991), it has no effect on DNA interstrand cross-link formation, suggesting that DNA covered by UvrA is essentially in B form. In contrast, UvrB bound at the damaged site, which produces a 19-bp footprint, drastically inhibits interstrand cross-link formation and instead itself becomes cross-linkable. Even though DNA-psoralen and RNA-psoralen cross-linking have been successfully used for probing nucleic acid structures for nearly 2 decades (see Cimino *et al.* (1985)), this is the first observation of psoralen-protein cross-linking. It is hoped that this method will aid in identifying the DNA-binding site of UvrB and other DNA-binding proteins.

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